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13. ABSTRACT ***(Maximum 200 Words)***

We have proposed the identification of junctionally transmitted growth control signals which we hypothesize to be responsible for the growth inhibition of tumor cells when in junctional communication with normal cells. We have genetically engineered human breast cancer cells to express connexin 43 under the control of a tetracycline-inducible promoter. When induced these cells regain junctional competence and their neoplastic potential is reduced in two important respects: one, when in communication with growth inhibited normal cells their proliferation is reduced; two, when these cells are cultured in suspension in soft agar, their colony formation is strongly reduced. Thus connexin 43 is acting as a tumor suppressor gene in these breast cancer cells. Increased junctional communication could thus lead to reduced progression of this disease. We have encountered significant problems in the molecular analysis of cells cultured in both situations. In co-culture, it is proving difficult to analyze one cell type without contamination by the second; in anchorage independent growth conditions, it is proving difficult to obtain sufficient cells for analysis without contamination with agarose. Both problems are being actively addressed.

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The data contained in this report constitutes unpublished data and has been marked "confidential".

Introduction:

We propose to chemically identify the junctionally transmitted signals which we hypothesize to be responsible for the observed growth inhibition of breast tumor cells when in junctional communication with growth inhibited normal cells. Our previous studies with murine cells have shown that when recently derived, neoplastically transformed fibroblasts were placed in junctional communication with growth inhibited fibroblasts, the transformed fibroblasts were arrested in G1 of the cell cycle. This cell cycle arrest strongly correlated with the degree of junctional communication (1). We lately extended these studies to human epithelial tumor cells in culture and demonstrated that the inducible expression of connexin 43, a gap junction family member expressed in normal epithelial cells but lacking in the carcinoma cells, connexin expression resulted in strong attenuation of the neoplastic phenotype. This was detected as a reduction in anchorage independent growth and a reduction in the ability to grow as xenografts in the nude mouse (2). Other investigators have also demonstrated reductions in connexin assembly or expression in neoplastic cells (reviewed in (3)). In several cases, this reduction in expression has been associated with an increase in DNA methylation, a method of gene silencing commonly employed by tumor cells to silence tumor suppressor genes (4). We have hypothesized that growth inhibitory signals can be transferred through gap junctions (5). Because of the physical constraints of the channel formed by a gap junction these inhibitory signals must be mediated by molecules or ions which are water-soluble and of a size below approximately 1000 daltons (6). Restoration of gap junction function could thus lead to the decreased proliferation of carcinogen-initiated cells thereby reducing their progression to fully transformed cells and it is of great interest that two classes of cancer preventive agents, the retinoids and carotenoids cause upregulated expression of connexin 43 in cells of epithelial and fibroblastic origin (7,8). Moreover, the chemical identification of the putative junctional transfer signal could offer new avenues for cancer therapy and perhaps prevention.

1/. Research accomplishments.

Technical objectives:

Task 1a. As previously reported, this goal has been achieved.

Task 1b, Developments of in vitro protocols for the delivery of the growth inhibitory signal from quiescent cells to junctionally competent breast cancer cells.

Growth in monolayer. To determine if the MDA breast cancer cell line produced any response to endogenous growth regulatory signals when allowed to junctionally communicate after connexin 43 induction, we plated cells at high density and monitored proliferation rates and cell cycle parameters under conditions of induction or when maintained with doxycycline. As previously reported cells became junctionally competent as judged by dye injection studies, however we could detect no change in cellular proliferation rates. We have previously reported that in one cell line, there was an increase in cells in the G2 portion of the cell cycle. However, we have been unable to reproduce these findings in subsequent studies of this line and other inducible lines, and we conclude, as was suggested by the reviewer of our prior progress report, that this conclusion was the result of abnormally low numbers of cells in the control, non-induced situation. It thus appears that the MDA breast carcinoma cells, are unable to either generate or respond to junctionally transmitted signal molecules. We reported a similar finding in the HeLa human cervical cancer cell line, which also failed to alter proliferation rates when forced into communication by induction of connexin 43 expression (2,7).

Growth in co-culture with quiescent normal cells. Even if the MDA cells can no longer produce growth inhibitory signals, they may well be able to respond to such signals if delivered through gap junctions. As reported in the previous year's annual report, connexin 43-induced MDA cells become junctionally competent when co-cultured with NRK cells and their ability to form colonies is significantly reduced. This inhibition was dramatically increased when we added an agent which increased intercellular cAMP levels. We have previously reported on the ability of cAMP modifying agents to increase heterologous gap junctional communication (9); an effect presumed to be due to the requirement for phosphorylation on the C-terminal region of connexin 43.

In an attempt to increase the degree of growth inhibition, we have co-cultured these MDA cells with other growth inhibited cells. These include: growth inhibited mouse 10T1/2 cells, human MCF-10a cells which represent an immortalized yet growth controlled mammary epithelial cell line, and with normal human fibroblasts. In all cases the MDA cells failed to make adequate contact with these growth controlled cells and formed discrete clusters of proliferating cells. Numerous techniques have been attempted to increase the interactions between these various cell types including; growth on matrigel coated dishes, growth on collagen coated dishes and treatment with agents that increase intercellular cAMP levels. In no case did these strategies increase the extent of growth inhibition of the MDA cells, and did not result in growth inhibition by any of the other cell types mentioned above. It should be noted that our previous work demonstrating the virtual complete suppression of cell cycle transit when transformed cells were in contact with growth inhibited normal cells, was conducted with very recently derived transformed cells which presumably had retained intact many of the growth inhibitory pathways necessary in cell cycle arrest (1). In contrast, the MDA cells utilized here represent a cell line which has

been extensively cultured and was derived from a highly metastatic human tumor. It seems likely that this extensive history of proliferation in the host and in cell culture conditions has led to the loss of many of the pathways that are required for efficient cell cycle inhibition.

Anchorage independent growth. We have now firmly established that induction of connexin 43 results in a greatly attenuated ability of these MDA cells to grow in an anchorage independent manner. As shown in Fig. 1 induction of connexin 43 by withdrawal of doxycycline causes a profound decrease in the ability of cells to form spheroids suspended in agarose. After induction, there was a total loss of formation of large colonies, and reduction from 514 medium-size colonies in the control situation to only 129 colonies in the induced situation. In terms of total colonies, the figure of 572 controls was reduced to 372 in the induced situation. As shown in the top left panel of Fig 1, when the parental MDA cells were plated in semi solid agarose the addition or subtraction of doxycycline had no effect on colony formation, clearly indicating that this is a connexin 43-specific event. The ability to grow in soft agar, i.e. anchorage independent growth, is a widely used indicator of neoplastic potential, and thus the suppression of growth caused by connexin 43 induction is significant in terms of the potential neoplastic properties of these cells.

While growth in semi solid agarose offers us an excellent assay with which to determine effects of connexin 43 expression on growth control, it also presents a formidable challenge in obtaining meaningful data which would identify molecular events induced in the cells as a consequence of connexin 43 induction. The difficulties here relate to problems in extracting cells in sufficient numbers, and uncontaminated by agarose, for us to perform of RT/PCR analysis of gene expression. These difficulties are twofold: first connexin 43 induction dramatically reduces the numbers of cells available for assay; second we have discovered that agarose is particularly difficult to remove from cells and contaminates nucleic acid extracts. To our knowledge, molecular assays have not before been reported in cells cultured in this matter. In an attempt to circumvent the problems associated with agarose contamination, we have grown cells in suspension in conventional media and denied their ability to attach to the culture dish by coating it with poly HEMA. However, in this situation, when cells did not proliferate to form spheroids as they did in agarose but remained as single cells without stable cell/cell contacts, no reduction in proliferation was observed. While this observation strongly supports the role of junctionally-mediated intercellular communication afforded by connexin expression, it did not solve our problem. We are currently evaluating the ability of enzyme treatment with agarase, as well as differential centrifugation as a means of obtaining uncontaminated cells.

Task 1c. Detection of the junctionally transferred signal in cancer cells by measurement of cell cycle related parameters.

Studies in co-cultured cells. The growth inhibition noted when cells were cultured with NRK cells could prove to be a useful assay for junctionally transmitted signals. In order to create a more quantifiable and rapid assay system, we have spent much time this last year attempting to measure the changes in cell cycle distribution in co-cultured MDA cells. Two strategies have been attempted in order to discriminate the MDA cells from the NRK cells: inducible MDA cells have been additionally genetically engineered to express a modified green fluorescent protein (EGFP); in a second approach, cells were immunostained with a human-specific antibody and counter stained with a FITC conjugated secondary antibody. By FACS analysis we were able to easily discriminate the two cell types on the basis of EGFP or FITC emission respectively. Unfortunately, when cells were additionally stained with the DNA specific dye propidium iodide, cross-talk between the red, propidium channel and the green EGFP or FITC channel resulted in poor discrimination between the cell types and we were thus unable to generate a cell cycle profile of the MDA cells. While this problem could be circumvented either by sorting of the MDA cells prior to DNA staining or by use of a blue-emitting DNA stain, we do not have access to either a cell sorter or FACS machine with a UV emitting laser.

One approach to overcome this problem has been the development of cells expressing high levels of EGFP. However, this approach was found to have the limitation that there was a strong correlation between EGFP emission and cell death, evidenced by increasing numbers of fragmented cells (Fig 2). Evidently strong over-expression of EGFP is lethal. Because of the importance of this assay, we are evaluating use of other DNA stains which may avoid this signal overlap problem.

Studies in anchorage independent cultured cells. In addition to the problems alluded to above regarding agarose contamination, a second problem concerns obtaining sufficient numbers of cells as a monodispersed suspension suitable for FACS analysis. The present we have been unable to obtain cells of sufficient quality i.e. as single cell suspensions without agarose contamination as judged microscopically. We are at present evaluating the potential use of digital analysis of microscopic images after DNA staining.

Task 1d Detection of molecular events induced by the junctionally transferred signal. In preparation for our successful isolation of cells which have been growth inhibited as a consequence of connexin 43 expression, we have assembled a panel of 10 probes for RT/PCR analysis. These probes have been chosen to represent proteins whose expression level is known to change with the transit from G1 to S-phase, believed to be the most likely checkpoint activated in communicating cells. Certainly, our previous studies with murine cells demonstrated that growth arrest was associated with G1 delay (1). Completion of these studies awaits our solving the cell contamination problems discussed above.

Tasks 2a-c. These have not yet been initiated because of the above-mentioned technical problems in obtaining a robust system for the assay of growth control signals.

Reportable outcomes:

Demonstration of strong inhibition of soft agar growth, an indicator of neoplastic potential, when MDA breast carcinoma cells are induced to express connexin 43.

Production of connexin 43 inducible MDA cells also expressing green fluorescent protein.

Conclusions:

Connexin 43 acts as a tumor suppressor gene in breast carcinoma cells decreasing growth in soft agar and reducing colony formation when in communication with growth-controlled normal cells.

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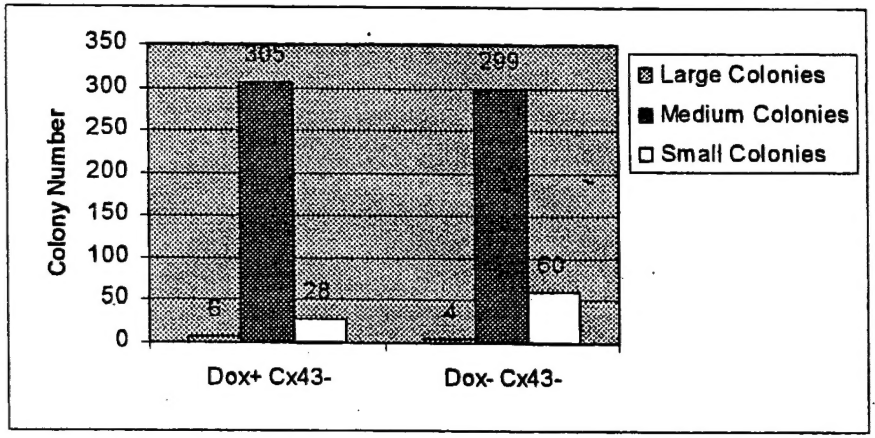
Appendices:

Figure 1. Connexin43 Expression Reduces Soft Agar Colony Formation in Connexin43-induced MDA-MB-435 Cells. One thousand cells were plated in 0.4% semi-solid agarose and cultures for 3 weeks. Colonies were then counted and scored for size. In the parental MDA cells the presence or absence of doxycycline did not alter growth, in contrast in the inducible cells, connexin43 induction lead to a dramatic reduction in total colonies and a proportionate increase in small colonies. In those colonies connexin 43 was induced as shown by immunofluorescence after staining with anti-connexin 43.

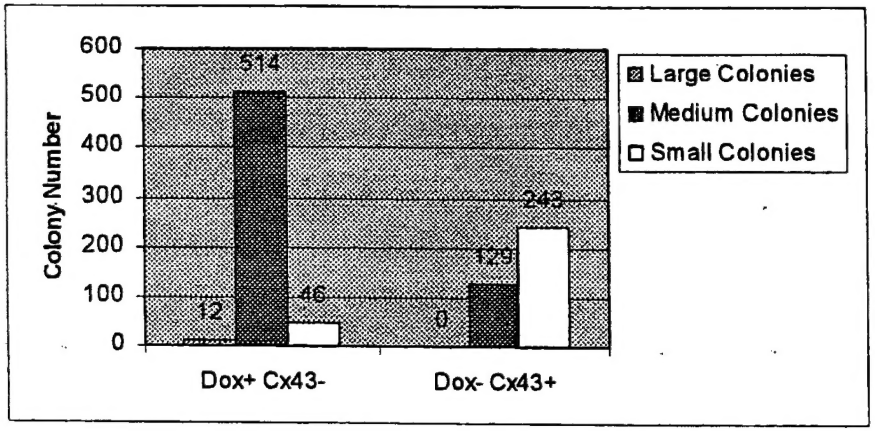
Figure 2. Overexpression of EGFP causes large increases in dead cells. Top panel: FACS analysis of inducible cells prior to EGFP transfection. FL1-H green fluorescence; SSCH, side scatter indicative of cell size. Middle panel; transfected clone expressing medium levels of EGFP; lower panel, clone expressing high levels of EGFP. Note increasing levels of cell fragments with increasing EGFP expression.

Connexin43 Expression Reduces Soft Agar Colony Formation in Connexin43-induced MDA-MB-435 Cells

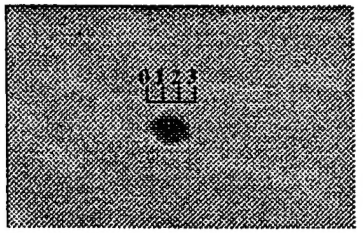
Parental MDA-MB-435 Cells



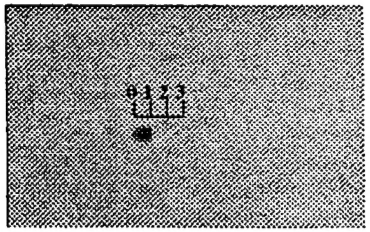
Connexin43 inducible MDA-MB-435



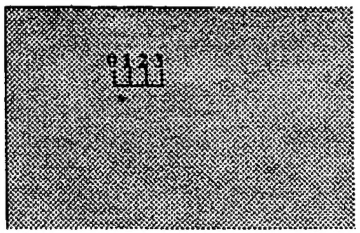
Phase Image of Colonies in Soft agar Assay



LargeColonies(>3)

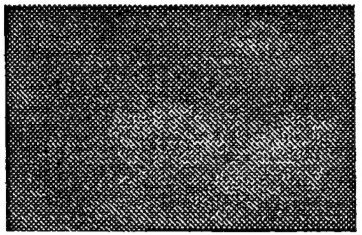


Medium Colonies (1-3)

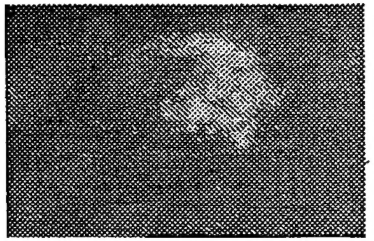


Small Colonies (<3)

Immunofluorescence Staining of Colonies in Soft agar Assay

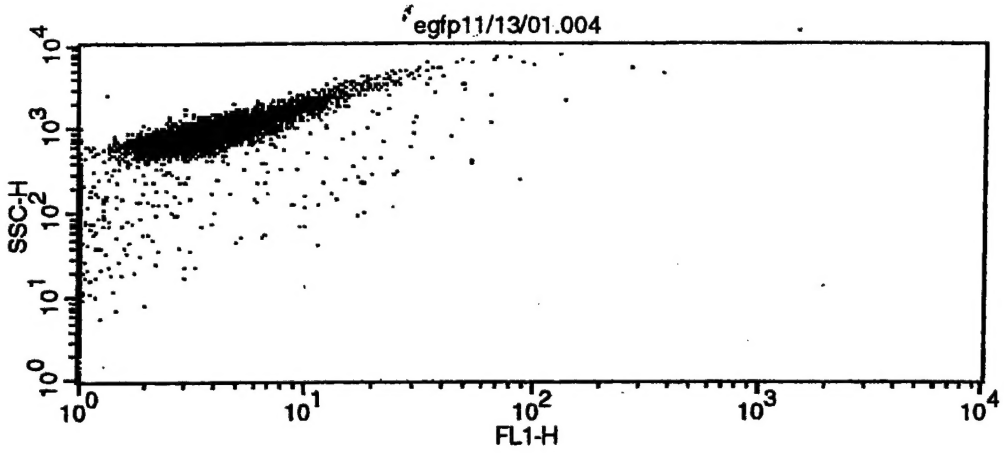


Connexin43-



Connexin43+

A



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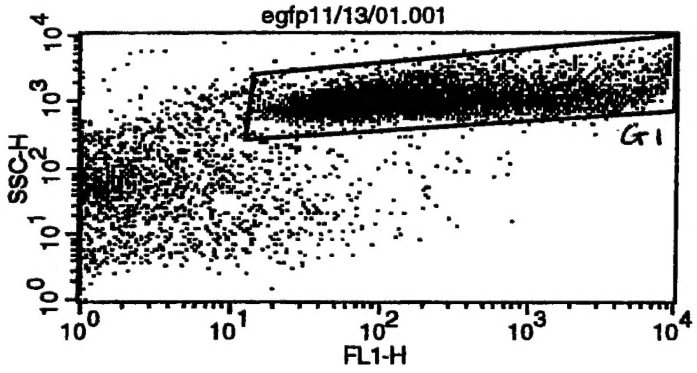

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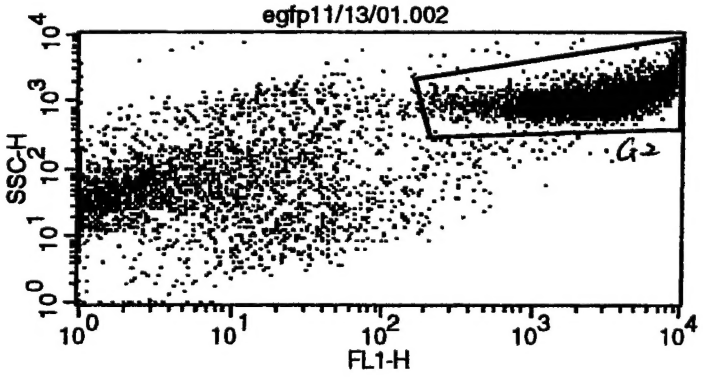

Figure 2. FACS analysis showing that overexpression of EGFP causes large increases in dead cells.